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ONCOLOGY

Expression of the SST receptor 2 in uveal melanoma is not a prognostic marker

Mariam Kouch-el Filali · Emine Kilic · Marleen Melis · Annelies de Klein · Marion de Jong · Gregorius P. M. Luyten

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Abstract
Introduction Uveal melanoma (UM) cells and neurohormone-producing cells both originate from the neural crest. Somatostatin receptors subtype 2 (SSTR2) are overexpressed in several tumors, often from neuroendocrine origin, and synthetic antagonists like octreotide and octreotate are being used as diagnostic or therapeutic agents. We investigated the SSTR2 expression in UM, and determined whether this expression was related to prognosis of the disease.
Materials and methods UM cell lines and fresh primary UM samples were tested for SSTR2 expression by autoradiography (AR) using 125I-Tyr3-octreotate. Furthermore, UM cell lines were analyzed for SSTR2 mRNA expression with quantitative real-time RT-PCR.
Results Using AR, cell-surface SSTR2 expression was demonstrated in two UM metastatic cell lines, but no expression was detected in three cell lines derived from primary UM. However, all primary and metastatic UM cell lines showed mRNA expression levels for SSTR2 using quantitative real-time RT-PCR. Only three of 14 primary UM demonstrated moderate SSTR2 expression, and this expression was not significantly associated with tumor-free survival or any tested prognostic factor.
Conclusions Based on the rare and low expression of SSTR2 found in primary UM specimens and in UM cell lines, we conclude that SSTR2 is not widely expressed in UM. Furthermore, SSTR2 expression was not associated with tumor-free survival and prognostic factors. Therefore SSTR2 is not suited as prognostic marker or therapeutic target in UM.

Keywords Uveal melanoma · Neurohormone · Somatostatin · Autoradiography

M. Kouch-el Filali · E. Kilic · G. P. M. Luyten
Department of Ophthalmology, Erasmus MC, Rotterdam, The Netherlands

M. Melis · M. de Jong
Department of Nuclear Medicine, Erasmus MC, Rotterdam, The Netherlands

A. de Klein
Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands

Present address:
M. Kouch-el Filali (✉) · G. P. M. Luyten
Department of Ophthalmology LUMC, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands
e-mail: m.el_filali@lumc.nl

Introduction

Uveal melanoma (UM) is the most common primary intraocular tumor, with an annual incidence of 0.7/100,000 in the Western population [1]. Although less than 2% of the patients have clinically detectable metastasis at presentation, 50% of all patients die due to metastatic disease. The median survival after the diagnosis of metastasis is extremely poor. Predictors of survival for UM patients have been identified in histologic cell type, tumor diameter, tumor location, age, gender [2, 3], and cytogenetic parameters. Loss of chromosome 3 is one of the most significant predictors for uveal melanoma-related deaths [4–6].

Most of the UM cases are treated by radiotherapy, thus material for histopathologic and cytogenetic examination has to be obtained by fine needle aspiration biopsy (FNAB) for example. Shields et al. showed that

FNAB provides adequate DNA for genetic analysis of UM using a microsatellite assay [7]. Early and long-term complications following intraocular FNAB are rare [8]. Nevertheless, one suspected clinical case of extrascleral recurrence has recently been reported after diagnostic intraocular transscleral FNAB [9]. Therefore concern about tumor seeding due to this diagnostic procedure still exists [10], and FNAB is consequently not used routinely yet.

Conversely, neurohormone receptors as markers could be detected non-invasively and safe using scintigraphy, without the need to take a biopsy.

During embryogenesis, neural crest cells migrate to the diencephalon and to the uvea, where they give rise to pigmented melanocytes. Neural crest cells are able to produce neurohormones like somatostatin (SST). SST inhibits the release of growth hormone and thyroid-stimulating hormone. Its actions are mediated by specific G protein-coupled receptors, which are located in specific target cells of the gastrointestinal tract, the peripheral nervous system and several blood vessels [11].

Moreover, SST could be involved in the inhibition of tumor growth [12, 13]. SST receptors (SSTR) have been detected in human neuroendocrine tumors [14–16], human lung tumors such as bronchial carcinoids [17] and gastro-entero-pancreatic tumors like insulinomas, gastrinomas, and ileal carcinoids [18]. SST analogues, like octreotide and octreotate, can be radiolabelled with radionuclides via a chelator and are currently being used in the diagnosis (^{111}In) or therapy (^{90}Y or ^{177}Lu) of patients suffering from SSTR-expressing tumors [19–22].

Due to the common origin of SST and UM cells, a relation or interaction may be found. A relation between eye tissue and neurohormones has already been explored in several studies [23–25]. In a previous study, Ardjomand et al. concluded that expression of SST receptors subtype 2 (SSTR2) in UM is correlated with a better ad vitam prognosis of the patients [26]. Our aim was to further investigate the expression of SSTR2 in UM, in order to identify specific membrane receptors for diagnostic imaging and therapeutic targeting. Primary specimens and UM cell lines, derived from primary or metastatic UM were analyzed using in vitro autoradiography and quantitative real-time RT-PCR techniques.

Materials and methods

Patient and tumor material

Informed consent was obtained prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki.

Fresh tumor tissue was obtained within 1 h after enucleation, according to a standardized protocol. An incision was made through the tumor, leaving the optic nerve intact. A sample was taken from the side opposite the optic nerve and divided into two; one part was processed for fluorescent in situ hybridization (FISH) and another part, as well as control tissues (rat brain, rat pancreas), were stored in liquid nitrogen for cryopreservation. Afterwards the remaining tissues were fixed in 4% paraformaldehyde overnight. Five-micrometer paraffin sections were cut on a microtome (Microm HM 335^{E}), and put on uncoated slides (Menzel Superfrost) humidified with sterile water for haematoxylin-eosin (HE) and periodic acid-Schiff (PAS) staining. Five-micrometer cryosections of frozen tumor samples and control tissues were cut on a cryostat (Jung CM3000; Leica, Meyer Instruments, Inc. Houston, USA), and mounted on coated slides. Subsequently, the slides were air-dried and stored at –80°C and processed as below (see under 'autoradiography'). Conventional histopathologic examination was performed on all tumors and confirmed the origin of each one.

Cell lines

Mel202, 92.1 and OCM-1, primary tumor-derived cell lines, were used as a model for human primary UM. OMM1 and OMM2.3, skin and liver metastases-derived cell lines, were used as a model for human UM metastasis. Rpe1 is a normal retinal pigment epithelium-derived cell line. OCM-1 was provided by Dr. J. Kan-Mitchell, Mel202 and OMM2.3 by Dr. B. Ksander, 92.1 by Dr. M.J. Jager and OMM1 and Rpe1 were established in our laboratory [27–31]. The SSTR-expressing CA20948 cell line originated from the solid CA20948 rat pancreatic tumor and served as positive control for autoradiography experiments since ocreotate detect both rodent and human SSTR2, of which the homology is more than 90% [19, 32]. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (GIBCO, Life Technologies, Paisley, UK). The cells were passaged once or twice a week using trypsin (0.05%) or trypsin + EDTA (0.02%). Cells were centrifuged at 1,000 rpm for 10 min in DMEM, resuspended in 0.1 M DPBS and used to prepare cytospins on coated slides (Menzel Superfrost Plus Menzel-Gläser, Braunschweig, Germany) humidified with DPBS using a cytofuge (Nordic, Tilburg, The Netherlands).

Fluorescent in situ hybridization analysis

Dual-color FISH was performed on uncultured tumor tissue, by using centromeres, locus-specific cosmids, P1, or YAC as probes for chromosomes 1, 3, 6, and 8, respectively, as described previously [33]. Seven probes

were used: p1–79 (mapped to chromosome band 1p36), P 3.5 (centromere 3), YAC 827D3 (3q24), cos85 (6p21), and cos52 (6q23) (all from Yusuke Nakamura, Tokyo, Japan) and D8Z2 (centromere 8) and ETO (8q22). The probes were validated on normal peripheral blood cell metaphase spreads, and ten metaphases were analyzed for each probe. Cutoff limits were less than 3%. The concentration for centromeric probes was 5 ng per slide; for cosmid, P1, and YAC probes, 50 to 75 ng per slide was used. After hybridization and washing, the slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in anti-fade medium (Dabco-Vectashield 1:1; Vector Laboratories, Burlingame, CA). Signals were counted in 300 interphase nuclei, according to the criteria of Hopman et al. [34]. Scoring for deletion (>15% of the nuclei with one signal) or amplification (>10% of the nuclei with three or more signals) was adapted from the available literature [35].

Autoradiography

^{125}I-DOTA-Tyr^3-octreotate (BioSynthema, St. Louis Mo, USA), that has a high affinity binding to SSTR2, was used for the autoradiography study [36]. The autoradiographic experiments were performed on cryosections and cytospins using 10^{-9}M or 10^{-10}M ^{125}I-DOTA-Tyr^3-octreotate, with or without a blockade of 10^{-6}M unlabelled octreotide (Novartis, Basel, Switzerland), to investigate SSTR2 specific binding as described in literature [14, 37]. After 1 h incubation at room temperature and rinsing with Tris buffer to remove the non-bound radioactive octreotate, dried cryosection, and cytospin slides were exposed to phosphor-imaging screens (Packard Instruments Co., Meriden, USA) in X-ray cassettes. After 24–72 h, the screens were read using a Cyclone phosphor imager and analyzed with OptiQuant 03.00 image processing system (Packard Instruments Co., Groningen, The Netherlands). Binding of ^{125}I-DOTA-Tyr^3-octreotate to cytospins and cryosections was expressed in digital light units (DLU)/mm^2. Net DLU/mm^2 represents binding of ^{125}I-DOTA-Tyr^3-octreotate (specific binding) minus non-specific binding in adjacent sections incubated with ^{125}I-DOTA-Tyr^3-octreotate plus 10^{-6}M octreotide. Net DLU/mm^2 was considered positive when >5,000 DLU/mm^2.

Quantitative real-time RT-PCR

SSTR2 mRNA expression was analyzed by quantitative real-time-reverse transcriptase–polymerase chain reaction (real-time RT-PCR). RNA was isolated using an RNeasy® Mini Kit (Qiagen, Valencia, USA). RNA samples were stored at –80°C until further processing. Approximately 1 μg of RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The 20-μl solutions obtained this way were diluted by adding sterile water up to 100 μl. In 96-wells, 2 μl of this solution was added to a 15-μl solution of iQ SYBR Green Supermix, forward and reverse primers (10 μM solutions) for SSTR2 or RPS-11 (control gene) and sterile water (volume ratio, respectively, 10:1:1:8). The primers had the following sequences: SSTR2 forward 5′-TGCTGGGTCTGCCTTTCTTGG – 3′, SSTR2 reverse 5′ – AGAAGATGCTGGTGAACTGATTGATG – 3′, RPS-11 forward 5′ – AAGCAGCCGACCATCTTTCA – 3′, RPS-11 reverse 5′ – CGGGAGCTTCTCCTTGCC – 3′ [38]. A quantitative analysis of the samples was then performed for SSTR2 and RPS-11 (control gene) expression by real-time RT-PCR in a MyiQ iCycler real-time PCR system (Bio-Rad, Hercules, CA). The PCR reaction settings were 95° for 5 min, then 40 cycles at 96° for 15 s and 60°C for 45 s. DNA melting peaks were acquired by measuring the fluorescence of SYBR Green during a linear temperature transition from 70°C to 97°C at 0.2°C each 10 seconds with accompanying software (Bio-Rad Laboratories BV). To correct the sample-to-sample variation when determining gene expression, an accepted method is to select a cellular housekeeping gene that serves as an endogenous control, against which the target gene expression levels can be normalized [39]. RPS-11 (ribosomal protein S11) is a housekeeping gene that has recently been introduced to normalize gene expression in UM cells [40].

Results

Patients and tumor material

In total, primary UM from 14 patients (ten male and four female, with a mean age of 63±10.1 years) were included in the study. Histopathologic analysis of HE and PAS stained paraffin sections was performed on all primary UM. The mean tumor diameter was 12.6±2.8 mm and their mean prominence was 8.5±2.0 mm. Epithelioid cells were found in ten of 14 cases, while in seven cases vascular loops and/or networks were detected. Furthermore, FISH analysis of all samples was performed; monosomy 3 as well as loss of chromosome 1p was found in 10 of 14 cases and gain of chromosome 8q was present in 7 of 14 cases. After a mean follow-up period of 36.1±13.0 months, five patients had developed distant metastases (Table 1).

Autoradiography

Fresh primary UM samples were tested for expression of SSTR2 by AR using ^{125}I-Tyr^3-ocreotate. In three of the 14 primary UM, a positive (albeit weak) binding was observed (Table 2). Statistical analysis with the Kaplan–Meier method and log-rank test did not show any significant

Table 1 Clinical, histopathologic, and genetic data of included patients

Sex (male/female)	10/4
Age (mean±SD, years)	63.0±10.1
Diameter (mean±SD, mm)	12.6±2.8
Prominence (mean±SD, mm)	8.5±2.0
Cell type (epithelioid/non-epithelioid)	10/4
Vascular loops and/or networks (yes/no)	7/7
Pigmentation (yes/no)	8/6
Metastasis (yes/no)	5/9
Monosomy 3 (yes/no)	10/4
Loss of 1p (yes/no)	10/4
Gain of 8q (yes/no)	7/7
Follow-up (mean±SD, months)	36.1±13.0

relation between SSTR2 expression and tumor-free survival (p=0.76). In the univariate and multivariate Cox-regression analysis, SSTR2 expression was not significantly associated with tumor-free survival or other important prognostic factors (cell type, vascular patterns, tumor diameter, prominence, monosomy 3, loss of chromosome 1p and gain of chromosome 8q).

UM cell lines were also tested for expression of SSTR2 by AR using ^{125}I-Tyr^3-ocreotate. All cell lines were tested in duplicate.

The positive control rat pancreatic tumor cell line (CA20948) showed a strong binding. Cell line 92.1 showed very low binding while none of the other primary UM derived cell lines (Mel202, OCM-1) or normal cell line (Rpe1) showed specific receptor binding. Both metastatic UM cell lines (OMM1, OMM2.3) showed high binding (Fig. 1, Table 3).

Table 2 Autoradiography results of primary uveal melanoma samples

	Metastasis[1]	Autoradiography[2] ^{125}I-Tyr^3-Ocreotate (SSTR2)
1	-	++
2	-	+
3	+	+
4	-	-
5	-	-
6	-	-
7	+	-
8	-	-
9	+	-
10	+	-
11	+	-
12	-	-
13	-	-
14	-	-
Control	NA[3]	++++

[1] Metastasis present: +, No metastasis:-
[2] Netto Digital Light Units/mm^2 : <5,000: –, 5,000–10,000: +, 10,000–50,000: ++, 50,000–100,000: +++, >100,000: ++++
[3] Not applicable

Quantitative real-time RT-PCR in cell lines

Expression of SSTR2 mRNA in UM cell lines was evaluated by real-time RT-PCR, with normalization of the expression levels to the housekeeping gene RPS-11. All

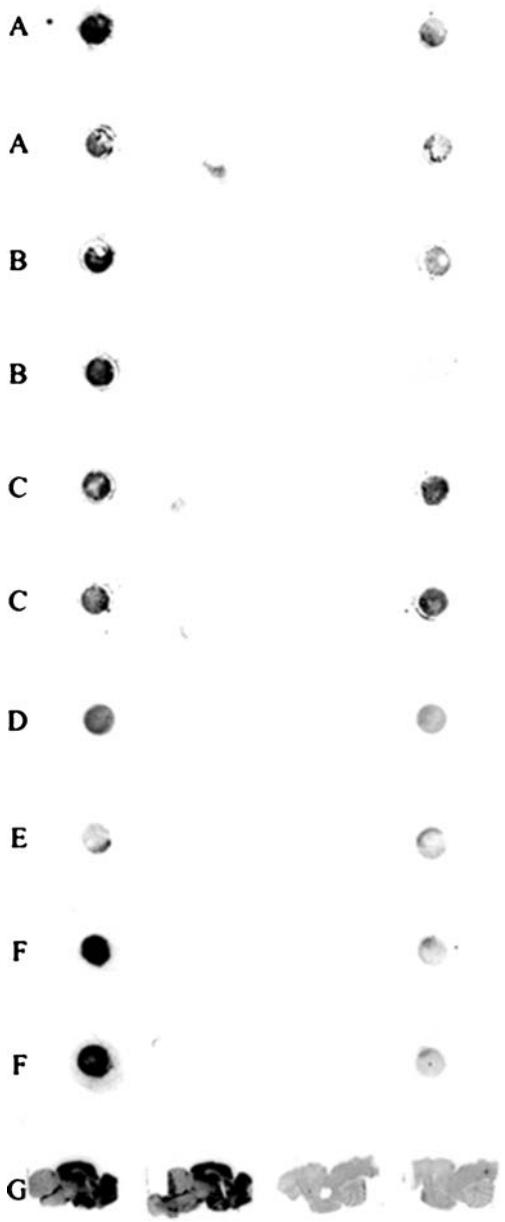

Fig. 1 Autoradiogram of cytospins of several uveal melanoma cell lines and controls demonstrating SSTR2 expression 1st column: ^{125}I-Tyr^3-ocreotate 2nd column: ^{125}I-Tyr^3-ocreotate + excess octreotide A. OMM2.3, B. OMM1, C. Mel202, D. OCM-1, E. Rpe 1, F. CA20948, G. Rat brain section (a few cell lines are shown in duplicate)

Table 3 Autoradiography results of uveal melanoma cell lines

Cell line		Autoradiography[1] ^{125}I-Tyr^{3}-Octreotate (SSTR2)
Primary	92.1	+
	Mel202	-
	OCM-1	-
Metastatic	OMM1	++
	OMM2.3	++
Normal Rpe[2]	Rpe1	-
Control	CA20948 [3]	++++

[1] Netto Digital Light Units/mm^2: <5,000: –, 5,000–10,000: +, 10,000–50,000: ++, 50,000–100,000: +++, >100,000: ++++
[2] Normal retinal pigment epithelium cell line
[3] Control cell line from the solid CA20948 rat pancreatic tumor

five UM cell lines tested expressed SSTR2 mRNA, but the level of expression was quite variable. OMM2.3 showed a two to four times higher expression level of SSTR2 mRNA compared to the other metastases derived UM cell line OMM1, and the primary UM cell lines 92.1 and Mel202. The other tested primary UM cell line OCM-1 showed a very low SSTR2 mRNA expression level (Fig. 2).

Discussion

Expression of somatostatin receptors in neuroendocrine tumors has been extensively investigated and led to the development of clinically relevant diagnostic and therapeutic strategies. Based on common embryonic origin, uveal melanoma could also be a candidate for such strategies, as many patients die from metastasized UM.

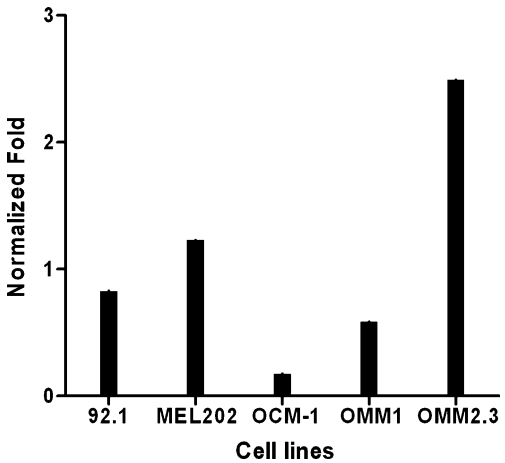

Fig. 2 Expression of SSTR2 mRNA in uveal melanoma cell lines. All five UM cell lines tested expressed SSTR2 mRNA. The expression levels were normalized to the housekeeping gene RPS-11. OMM2.3 shows a two to four times higher expression level of SSTR2 mRNA compared to OMM1, 92.1 and MEL202. Primary UM cell line OCM-1 had a very low expression level, and can therefore not be compared to the other UM cell lines

In this study, SSTR2 expression was studied in 14 primary UM tumor samples and several different UM cell lines, either derived from primary or from metastasized UM tumor samples. Primary tumors were tested at the protein level using in vitro autoradiography, cell lines were additionally analyzed on mRNA level with quantitative real-time RT-PCR. In only three primary specimens moderate SSTR2 expression was demonstrated. One of these was a metastasized UM, while four other samples of metastasized UM did not express SSTR2.

Furthermore, the relation between SSTR2 expression, tumor-free survival and prognostic factors was studied, although the number of samples we used was too small to perform reliable statistical analysis. With the data obtained thus far, no significant correlation between SSTR2 expression and tumor-free survival or any other important prognostic parameter could be found. Conversely, Ardjomand et al. detected SSTR2 expression in nearly all of 25 tested samples, using immunohistochemistry on paraffin embedded UM tissue. When the prognosis of these 25 patients was compared with the expression level of SSTR2 in uveal melanoma tissue, a positive correlation was found between high SSTR2 and a better ad vitam prognosis [26].

Since Ardjomand et al. used a different approach and techniques to determine SSTR2 expression, it is difficult to compare our results. An explanation for the differences could be that we used cryosections instead of paraffin sections, in antibody versus peptide targeting. Furthermore, in immunohistochemistry it is possible to discriminate positive staining in individual cells by using antibodies for specific subtypes of somatostatin receptors. In our AR experiments, we analyzed tissue cryosections and a peptide analogue that can be applied in vivo for PET or SPECT imaging, after labelling with an appropriate radionuclide. In AR a certain threshold had to be reached before tissue could be identified as positive; however, this threshold is low due to the high sensitivity of the phosphor imaging read out system. For in vivo imaging this threshold would be much higher. Most of the tumors tested by Ardjomand et al. contained between 11 and 80% of SSTR2 expressing tumor cells. If the percentage of SSTR2 expressing tumor cells in our tested specimens was within this range, these should have been detected using in vitro autoradiography. Furthermore, Ardjomand et al. also argued the diagnostic value of SSTR expression, since in only two of four patients with UM in their study the affected eye could be visualized by octreotide scintigraphy.

Besides primary UM samples, UM derived cell lines, originating either from primary or from metastasized UM tumors, were tested for SSTR2 expression. By using the in vitro AR technique with cytospins of UM cell line cells, it was found that three primary UM melanoma cell lines showed almost no specific binding of ^{125}I-Tyr^{3}-octreotate,

whereas both metastatic UM cell lines showed strong binding. This implied that expression of SSTR2 at the protein level could be associated with a bad prognosis and development of metastasis. This clear SSTR2 expression of the metastatic UM cell line OMM2.3 correlated with a four-times-higher expression level of SSTR2 mRNA compared to the primary UM line 92.1 in quantitative real-time RT-PCR. The primary UM cell line Mel202 also showed a moderately high SSTR2 mRNA expression, but apparently this mRNA was not transcribed to protein. The correlation between SSTR2 expression at mRNA and protein level seems to be good in cell lines derived from metastasized UM, but less in lines from primary UM. Furthermore, there is a discrepancy between the SSTR2 expression in cell lines compared to that in primary and metastasized uveal melanoma specimens, in which only a small percentage of low SSTR2 expressing samples was found. Expression of high SSTR2 levels might be favorable for deriving an in vitro growing cell line from an UM biopsy. Thus cell lines might not be really representative for in vivo circumstances [41].

Considering the low SSTR2 expression of the primary UM samples, we conclude that imaging with somatostatin analogues to perform scintigraphy is not feasible in uveal melanoma patients.

Although FNAB has been shown to accurately demonstrate genetic and histologic prognostic factors in the obtained tissue [7], finding another tumor-specific (neurohormone) receptor that can be used as an in vivo target for diagnosis (and therapy) is still an attractive, non-invasive option to improve the diagnosis of (metastasized) UM using specific radiolabelled peptide analogues. Metastasis is the single leading cause of death of patients with UM. Kaplan–Meier estimates of 5-year melanoma-related mortality range from 26 to 32% [42]. The first site of metastasis is the liver in approximately 90% of patients, but later spread to the lungs, bone, and skin occurs frequently [43–45]. It remains unknown to which extent current treatments for metastasis actually prolong survival compared to no treatment at all [46, 47]. It is proposed that patients who develop clinical metastases from uveal melanoma often harbor micro metastases for years [48]. Targeting tumor-specific receptors might be used to treat these (micro) metastases in future when specific analogues are radiolabelled with therapeutic β-emitting radionuclides like ^{177}Lu.

Further investigation targeting other neural-crest derived hormone receptors may thus reveal new options. Several studies have shown that vasoactive intestinal peptide (VIP) and pituitary adenylate-cyclase-activating polypeptide (PACAP) have tumor-growth promoting activities in breast cancer and neuroblastoma for example. Furthermore, VIP and PACAP antagonists demonstrated growth-inhibitory properties [49–52]. VIP receptors have been found to be ubiquitous expressed in all ocular tissues, with highest concentrations occurring in the choroid of several different mammals [23]. Most UM are strongly pigmented. Alpha-melanocyte-stimulating hormone is primarily responsible for the regulation of pigmentation and could therefore be linked to UM [53].

In conclusion, additional exploration of neurohormone receptors is needed to identify a specific membrane receptor to be used in diagnostic imaging and therapeutic targeting.

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Conflict of Interest Statement None

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